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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5) 10/030404

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/14133	23 May 2000	24 May 1999

TITLE OF INVENTION
HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES

APPLICANT(S) FOR DO/EO/US
Sanjay Tyagi, Ph.D., Fred R. Kramer, Ph.D. and

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other documents or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    - ☒ References
    - ☒ PTO Form 1449
    - ☒ International Search Report
    - ☒ Copy of published PCT
    - ☐ WO 00/71740 A1

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I hereby certify, under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, DC 20231.

Francisco Robles

Jan 4-2002 (Date of Deposit) Francisco Robles (Signature) (Typed Name of Person Signing)

U.S. APPLICATION NO. (IF KNOWN)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/030404		07763-044001

17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)- (5)):

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1040**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$740**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$710.00
Surcharge of **$130** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$130.00

Claims	Number Filed	Number Extra	Rate	
Total Claims	4 - 20 =	0	x **$18**	$0.00
Independent Claims	1 - 3 =	0	x **$84**	$0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ **$280**	$0.00
TOTAL OF ABOVE CALCULATIONS =				$840.00
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$420.00
SUBTOTAL =				$0.00
Processing fee of **$130** for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))				$0.00
TOTAL NATIONAL FEE =				$0.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$0.00
TOTAL FEES ENCLOSED =				$420.00
Amount to be refunded:				$
Charged:				$

a. ☒ A check in the amount of $420.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 06-1050 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

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HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES

This invention relates to antisense oligonucleotides and their therapeutic use.

Background of the Invention

Antisense oligonucleotides are therapeutic agents. Conventional antisense oligonucleotides are linear oligonucleotide sequences that are designed to bind to target sequences in messenger RNAs and thereby inhibit the translation of the messenger RNA into its encoded protein, or initiate a chain of events that causes the degradation of the messenger RNA with the effect that its encoded protein cannot be synthesized. The therapeutic use of antisense oligonucleotides may be hampered by poor specificity. For some applications of antisense oligonucleotides, the sequence of each messenger RNA is widely different from other messenger RNAs, and specificity is not a limitation. However, that is not always true. In some diseases the translation of a "wild-type" messenger RNA does not cause a pathogenic condition, but if a particular nucleotide substitution is present in that messenger RNA, then its expression does cause a pathogenic condition. It is desirable to be able to use an antisense oligonucleotide to inhibit the expression of such an RNA. Typically, the mutant messenger RNA is associated with cells that are cancerous. For example, the human *ras* gene becomes cancer-inducing by the acquisition of a single nucleotide point mutation within its coding sequence (Monia et al., 1992). In this and similar situations, it is important that the antisense oligonucleotide bind to the cancer-causing variant of the *ras* messenger RNA, but that it not bind to the normal *ras* messenger RNA that is present in healthy cells.

Relatively long oligonucleotides are used as antisense agents in order that they form strong hybrids and select against unrelated messenger RNAs. These agents do not discriminate in practice against single nucleotide differences. Shortening the length of the antisense oligonucleotide to improve its specificity for the mutant target sequence as compared to the almost identical wild-type sequence is not very useful,

because this weakens the hybrids that it forms, and as a result it is not able to inhibit the expression of mutant messengers RNA.

The present invention markedly improves the specificity of antisense oligonucleotides and antisense therapy.

Summary of the Invention

This invention includes modified antisense oligonucleotides having increased specificity. The antisense oligonucleotides of this invention, when not bound to target, assume a structured hairpin configuration comprising a single-stranded loop and a double-stranded stem. The loop is complementary to the target. Interaction of the loop to the target results in the formation of a loop-target hybrid, which causes the stem to unwind, or dissociate. Antisense oligonucleotides according to this invention are able to discriminate between an intended target messenger RNA and another messenger RNA differing from the target by a single nucleotide substitution.

Antisense oligonucleotides according to this invention have an internal sequence flanked by a pair of sequences that are complementary to one another and hybridize to one another under conditions of use in the absence of target messenger RNA. We refer to the three sequences as the 5' arm, the loop, and the 3' arm. Hybridization to the arms to one another creates a double-stranded region, or stem. Hybridization of the loop sequence to its perfectly complementary target sequence causes the stem to dissociate, or unwind. This hairpin structure, which is not possessed by conventional linear antisense oligonucleotides, markedly improves the ability to discriminate between two messenger RNAs that differ by a single nucleotide.

This invention also includes therapeutic use of these hairpin antisense oligonucleotide, which may be administered in the conventional fashion already known for linear antisense oligonucleotides, for example, by encapulating the agents in liposomes that fuse with all membranes and thereby deliver there contents into cells. Antibodies anchored on liposomes can target the liposomes to cancerous

tumors. Administration is in a therapeutically effective amount by which we mean an amount that is capable of producing a medically desirable result in a treated mammal, for example, a human patient.

Description of the Preferred Embodiments

The use of extremely specific hairpin antisense oligonucleotides that selectively inhibit the expression of a pathogenic gene, without inhibiting the expression of a related gene that differs from it by only a single nucleotide substitution, is based on the finding that the presence of the hairpin stem enhances the specificity of the probe sequence located in the loop of the hairpin structure. Hairpin antisense oligonucleotides are simple to design. The arm sequences of hairpin antisense oligonucleotides can be chosen independently of the identity of the target sequence. Only the loop portion of the hairpin antisense oligonucleotide needs to be complementary to the target. The loop sequences in hairpin antisense oligonucleotides are sufficiently long to be specific to a chosen sequence and relatively long as compared to the arms such that hybridization of the loop sequence alone drives the opening of the hairpin stem. The length of the loop sequence in a hairpin antisense deoxyriboligonucleotide according to this invention ranges from 7 to 30 nucleotides, preferably 10 to 25 nucleotides and more preferably 15-25 nucleotides, whereas the length of the arm sequence ranges from 3 to 8 nucleotides, with the loop sequence always being longer than the stem sequence. The specificity of hairpin antisense oligonucleotides increases as the length (or GC content) of the arm sequences is increased. Whether a particular hairpin antisense oligonucleotide actually exhibits the desired level of specificity can be tested *in vitro* by labeling the hairpin antisense oligonucleotide with terminal interactive labels according to the methods of Tyagi and Kramer (1996) and then detecting hybridization by observing the increase in fluorescence intensity. A hairpin antisense oligonucleotide will bind to a perfectly complementary target sequence in a messenger RNA, that is, a target perfectly complementary to the loop, but will not bind to a sequence in a messenger RNA that differs from the target sequence by a single nucleotide substitution, and, thus, is not perfectly complementary to the loop.

Another useful aspect of hairpin antisense oligonucleotides is that because they are in the form of a hairpin that possesses a double-stranded stem, they are naturally more resistant to cellular nucleases than conventional linear antisense oligonucleotides.

Hairpin antisense oligonucleotides of the present invention can contain deoxyribonucleotides, ribonucleotides, peptide nucleic acids (PNA), other modified nucleotides, or combinations of these. Modified nucleotides may include, for example, 2'-O-methylribonucleotides or nitropyrole-based nucleotides. Modified internucleotide linkages may also be included, for example phosphorothioates. The advantage of using such modifications for a particular application will be apparent to persons familiar with the art. In particular, hairpin antisense oligonucleotides constructed from modified nucleotides may form stronger hybrids than if the hairpin primers were constructed from deoxyribonucleotides, thus enabling structured target sequences (such as those that occur in messenger RNAs) to be more easily accessed. In addition, hairpin antisense oligonucleotides constructed from modified nucleotides, or that contain modified internucleotide linkages, will resist degradation by cellular nucleases, rendering them more effective as therapeutic agents.

Hairpin antisense oligonucleotides according to this invention may have an arm that is at least partially complementary to the intended target. However, it must open when tested against a target consisting of only the perfect complement of the loop.

Example

We have designed a highly specific hairpin antisense oligonucleotide that binds to a mutant carcinogenic form of *ras* messenger RNA, but does not bind to the wild-type form of *ras* messenger RNA. These two RNAs differ from one another by a single nucleotide substitution within codon 12 (Monia et al., 1992). This embodiment is made of deoxyribonucleotides. The sequence of the hairpin antisense oligonucleotide is 5'-CGCTGGCCCGCGGCAGCCACACCCCAGCG-3', where underlines identify the arm sequences that hybridize to each other, the 5' arm being the sequence CGCTGG. In the absence of target strands that are complementary to the single-stranded loop, the two arms hybridize to one another to form a stem. If the stem sequences had not been added to the loop sequence in this antisense oligonucleotide, it would not have sufficient capacity to discriminate between wild-type *ras* messenger RNA and mutant *ras* messenger RNA (Monia et al., 1992). However, the hairpin antisense oligonucleotide is much more specific for its intended target sequence because the sequence in the loop must initiate the binding of the oligonucleotide to the target sequence, and this interaction is much more specific than the binding of a conventional linear antisense oligonucleotide to the same target sequence because the interactive sequence in the loop is embedded within a hairpin stem. By only binding to mutant *ras* RNA, hairpin antisense oligonucleotides selectively inhibit the growth of cells that express the mutant messenger RNA (cancer cells), and do not inhibit the growth of cells that express the wild-type messenger RNA (healthy cells).

References

Monia, B. P., Johnston, J. F., Ecker, D. J., Zounes, M. A., Lima, W. F., and Freier, S. M. (1992) Selective inhibition of mutant Ha-ras mRNA expression by antisense oligonucleotides. J. Biol. Chem. 267, 19954-19962.

Tyagi, S., and Kramer, F. R. (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303-308.

Tyagi, S., Bratu, D. P., and Kramer, F. R. (1998) Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. 16, 49-53.

We claim:

1. An antisense oligonucleotide consisting of a central loop sequence that is complementary to a selected messenger RNA target sequence and that is flankded by 3' and 5' arm sequences that are complementary to one another, wherein in the absence of said target sequence said oligonucleotide assumes an hairpin structure having a double-stranded stem, wherein interaction of said loop sequence with said target sequence causes dissociation of said stem, and wherein interaction of said loop sequence with a sequence complementary thereto except for a single nucleotide does not cause said stem to dissociate.

2. The antisense oligonucleotide of claim 1 comprising nucleotides selected from the group consisting of deoxyribonucleotides, ribonucleotides, peptide nucleic acids, 2'-0-methylribonucleotides and nitropyrole-based nucleotides.

3. The antisense oligonucleotide of claim 1 comprising modified internucleotide linkages.

4. A therapeutic method comprising administering to a patient an antisense oligonucleotide according to claim 1.

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(81) Designated States *(national)*: AU, CA, JP, US.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES

(57) Abstract: This invention relates to hairpin antisense oligonucleotides and their therapeutic use.

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled HIGH SPECIFICITY HAIRPIN ANTISENSE OLIGONUCLEOTIDES, the specification of which:

[] is attached hereto.
[] was filed on _____ as Application Serial No. _____ and was amended on _____.
[x] was described and claimed in PCT International Application No. US00/14133 filed on May 23, 2000 as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119(e)(1) of any United States provisional application(s) listed below:

U.S. Serial No.	Filing Date	Status
60/135,560	05/24/1999	Abandoned

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Serial No.	Filing Date	Status
US00/14133	05/23/2000	Pending

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Application No.	Filing Date	Priority Claimed
			[] Yes [] No

Combined Declaration and Power of Attorney

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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